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Report

The DNA Glycosylase/Lyase ROS1 Functions in Pruning DNA Methylation Patterns in *Arabidopsis*

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Summary

The *Arabidopsis* DNA glycosylase/lyase ROS1 participates in active DNA demethylation by a base-excision pathway [1, 2]. ROS1 has been shown to be required for demethylating a transgene promoter [1]. To determine the function of ROS1 in demethylating endogenous loci, we carried out bisulfite-sequencing analysis of several transposons and other genes in the *ros1* mutant. In the wild-type, although CpG sites at the majority of these loci are heavily methylated, many of the CpXpG and CpXpX sites have low levels of methylation or are not at all methylated. However, these CpXpG and CpXpX sites become heavily methylated in the *ros1* mutant. Associated with this increased DNA methylation, these loci show decreased expression in the *ros1* mutant. Our results suggest that active DNA demethylation is important in pruning the methylation patterns of the genome, and even the normally "silent" transposons are under dynamic control by both methylation and demethylation. This dynamic control may be important in keeping the plant epigenome plastic so that it can efficiently respond to developmental and environmental cues.

Results and Discussion

DNA methylation is an epigenetic mark in plants, most animals, and some fungi [3–11]. In plants, DNA cytosine methylation can occur in any sequence contexts, i.e., CpG, CpXpG, and CpXpX (X is A, T, or C). The Dnmt3 subfamily of DNA methyltransferases, DRM1 and DRM2, functions in de novo methylation. Maintenance methylation at CpG sites is carried out by the Dnmt1 ortholog, MET1, and CHROMOMETHYLASE3 (CMT3) is responsible for CpXpG methylation [5]. DNA demethylation can occur either passively during DNA replication or actively in the absence of DNA replication [12]. In *Arabidopsis*, recent genetic and biochemical studies demonstrated that two bifunctional DNA glycosylase/lyases, ROS1 and Demeter, function as DNA demethylases [1, 2, 13, 14]. ROS1 can specifically recognize methylated DNA substrate. Its glycosylase activity removes the 5-methylcytosine base, and its lyase activity nicks the DNA backbone at the abasic site by a β,δ elimination mechanism [2]. Then, an unmethylated cytosine nucleotide is added through the actions of other enzymes in the DNA repair pathway [12]. Loss-of-function mutations in *ROS1* result in hypermethylation of the *RD29A* promoter and silencing of the *RD29A*-promoter-driven luciferase transgene [1]. Demeter has largely similar biochemical properties as ROS1, although its role is restricted to the two central cells of the female gametophyte where it is specifically expressed [13].

Transposable elements are major constituents of complex genomes of plants and animals. DNA methylation has been suggested to function in silencing transposons and thus providing genomes immunity against transposable elements [11]. Consistent with this notion, reduced DNA methylation and increased expression of transposable elements were observed in mutants defective in DNA methyltransferases or in RNA silencing or other components that regulate the methyltransferases [3, 5, 6, 11, 15]. To assess the potential role of active DNA demethylation in shaping the methylation patterns of the genome, we compared the wild-type and *ros1* mutant plants in the methylation status and expression level of the following representative transposable elements: the gypsy-class LTR (long-terminal repeat) retroelement *AtGP1*, non-LTR retroelement *AtLINE1-4*, short interspersed nuclear retroelement *AtSN1*, and MULE DNA transposon *AtMU1* [16]. In addition, *MEA-ISR*, a subtelomeric repeat sequence that is present downstream of the *MEA* gene, and *FWA*, which is under control of a SINE element in its 5′ region [17], were chosen for analysis.

Bisulfite sequencing analysis showed that the CpG sites at these loci are heavily methylated in the wild-type (Figure 1A). In the *ros1* mutant, there is a slight increase in CpG methylation. Methylation levels at CpXpG sites in the loci are much lower than at CpG sites. For *AtGP1*, *AtMU1*, *AtLINE1-4*, and *FWA*, there are substantial increases in CpXpG methylation in the *ros1* mutant compared to the wild-type (Figure 1A). Interestingly, CpXpG methylation is relatively higher for *AtSN1* and *MEA-ISR*, and the *ros1* mutant showed slightly lower levels compared to the wild-type.

CpXpX sites are more numerous than CpG or CpXpG sites at the loci examined. In the wild-type, CpXpX methylation levels for all the loci are the lowest compared to the CpG or CpXpG methylation. For all the loci except *MEA-ISR*, the CpXpX methylation levels increased in *ros1* (Figure 1A). This increase is most dramatic for *AtGP1*, where the level more than tripled in *ros1*. The DNA methylation levels at *AtGP1*, *AtMU1*, and *AtLINE1-4* were also tested by methylation-sensitive PCR with McrBC, which preferentially cuts methylated DNA. Higher levels of methylation result in increased McrBC digestion and consequently reduced amplification by PCR [16]. The results (Figure 1B) further support that *AtGP1*, *AtMU1*, and *AtLINE1-4* have higher levels of methylation in *ros1* than in the wild-type (Figure 1B).

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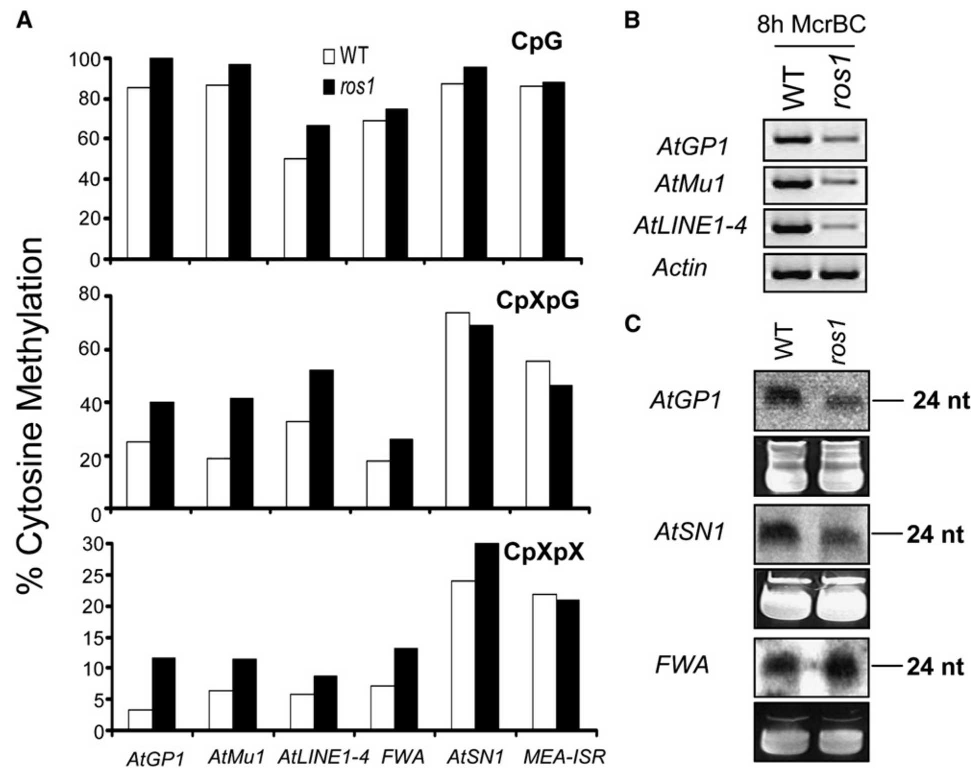

Figure 1. Comparison of DNA Methylation and siRNA Levels between the Wild-Type and the *ros1* Mutant

(A) Analysis of CpG (top), CpXpG (center), and CpXpX (bottom) methylation by bisulfite sequencing. The sequences analyzed are indicated at the bottom. X could be A, T, or C.

(B) DNA methylation analysis by McrBC PCR. McrBC-digested genomic DNA was amplified by PCR with primers for the indicated transposons. Input DNA was normalized for each genotype with *actin* primers.

(C) Detection of siRNAs in the wild-type and *ros1*. The size marker (24 nucleotides) is indicated. Ethidium bromide-stained gels corresponding to tRNA and 5S rRNA are shown at the bottom as the loading control.

For the *RD29A*-promoter-driven luciferase gene, the hypermethylation and silencing in *ros1* mutant plants are dependent on small-interfering RNAs (siRNAs) from the transgene *RD29A* promoter [12]. The *ros1* mutation does not affect the level of *RD29A* promoter siRNAs [1]. We examined the siRNAs from *AtGP1*, *AtSN1*, and *FWA* by Northern blot analysis and found that their levels are not increased in the *ros1* mutant (Figure 1C). The results indicate that the increased DNA methylation in *ros1* mutant plants is not a result of higher levels of siRNAs. Furthermore, the increases in largely non-CpG methylation at the loci in *ros1* do not appear to be causing more siRNAs to be produced through positive feedback regulation.

An examination of the detailed methylation sites at the transposons and *FWA* (Figure 2; see Figure S1 in the Supplemental Data available with this article online) revealed interesting patterns. For example, at *AtGP1*, all CpG sites are already highly methylated in the wild-type, whereas several CpXpG sites and most CpXpX sites are not methylated. However, many of these unmethylated sites become methylated in *ros1* mutant plants (Figure 2A). Although less dramatic, this change in methylation pattern in *ros1* is also true for *FWA* (Figure 2B), *AtMU1*, and *AtLINE1-4* (Figure S1). For *AtSN1* and *MEA-ISR*, most of the non-CpG sites are already methylated in the wild-type, and they showed relatively little or no increase in *ros1* mutant plants (Figure S2). These results suggest that ROS1 plays an important role in erasing methylation at many of the CpXpG and CpXpX sites in some loci.

RT-PCR [16] was carried out for determining the effect of *ros1* mutation on the expression of these loci. We found that *AtGP1*, *AtMU1*, *AtLINE1-4*, and *FWA* have a substantially lower expression in the *ros1* mutant than in wild-type plants (Figure 3A). For the transposon *AtSN1*, there appears to be a slight decrease in expression in *ros1*. Real-time PCR analysis was also done for *AtGP1*, *AtMU1*, and *AtLINE1-4*, and the results (Figure 3B) confirmed that these loci have less expression in *ros1* mutant plants. Recently, Huettel et al. [18] identified a number of endogenous targets of RNA-directed DNA methylation by differential-expression analysis. We tested the expression of several of these targets by real-time PCR and found that the expression of *At1g21310* and *At1g76930* (Figure 3C) but not *IG/LINE*, *IG2*, *IG5*, and *L18* (not shown) is consistently lower in *ros1* plants. In addition, real-time PCR analysis revealed a decreased expression of *AtCOPIA4* and 45S rDNA in the *ros1* mutant (Figure 3C). Thus, the effect of *ros1* mutation is clearly not limited to *AtGP1*, *AtMU1, AtLINE1-4*, or *FWA*. To identify more genes that show reduced expression in *ros1*, we carried out a microarray experiment by using Affymetrix *Arabidopsis* ATH1 Genechips. Out of the candidate genes showing at least a 2-fold-lower expression in *ros1* compared to wild-type seedlings

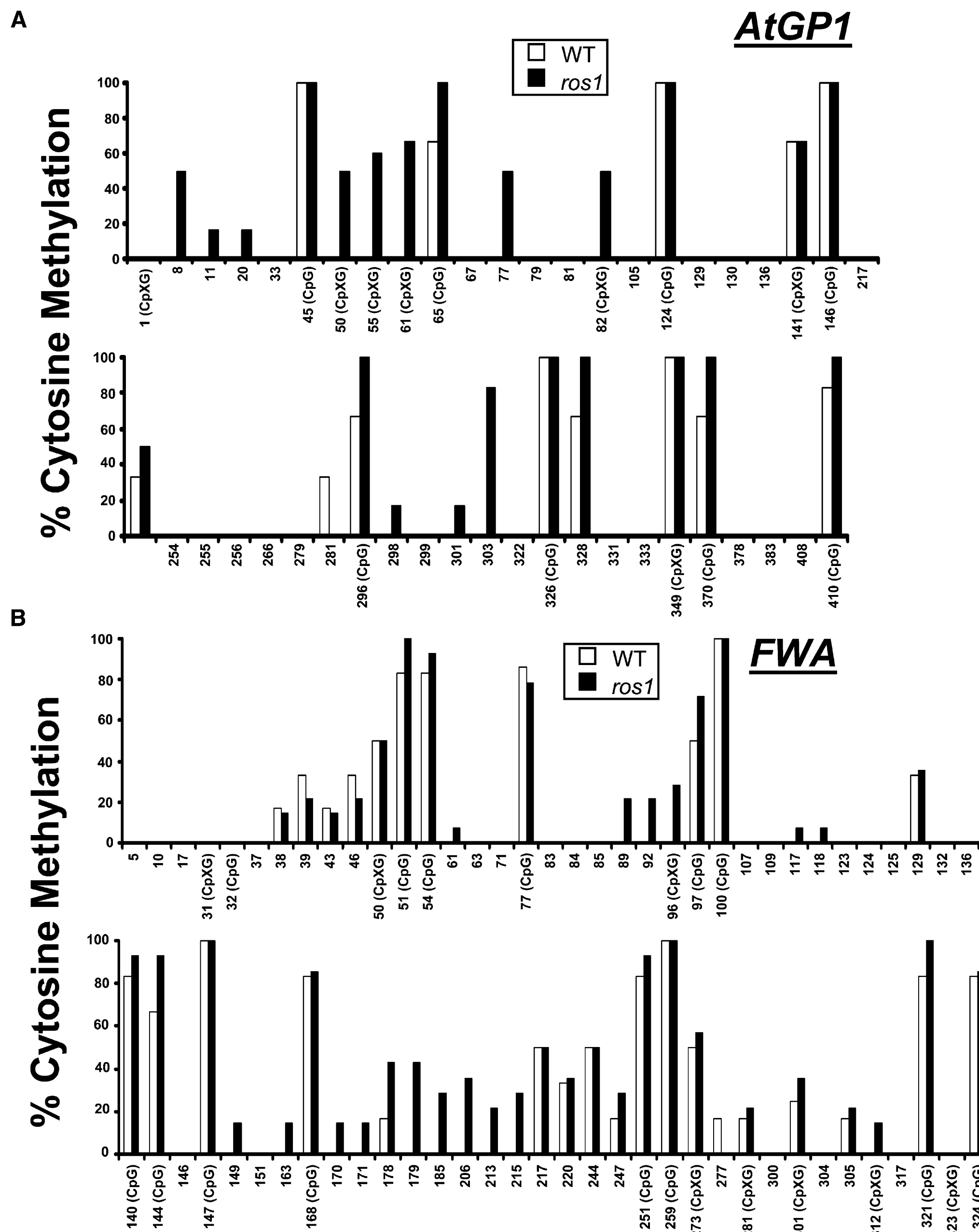

Figure 2. Detailed Cytosine Methylation Profiles Revealed by Bisulfite Sequencing

Cytosine positions and sequence contexts (CpG and CpXpG; CpXpX not indicated) are indicated on the x axis. (A) shows *AtGP1*. (B) shows *FWA*.

(Table S1), eight (*At2g41260*, *At5g46900*, *At5g44420*, *At5g51720*, *At2g33830*, *At5g01660*, *At4g18650*, and *At1g29460*) were selected for further analysis by real-time PCR. Seven of these were confirmed to have lower levels of expression in *ros1* seedlings (Figure 3C and Figure S3). Consistent with our previous report [1] and the above real-time PCR results, the microarray experiment showed an approximately 3- and 2-fold-lower expression of the *RD29A/COR78* and *At1g21310* genes (Table S1), respectively, in *ros1* than in wild-type seedlings. However, it appears that the microarray experiment did not identify all genes with lower expression in the *ros1* mutant because it did not detect a substantial expression change of the *At1g76930* gene, although this gene was consistently found to have a lower expression in *ros1* by real-time PCR assays (Figure 3C).

Bisulfite sequencing was carried out for assessment of the methylation status of 45S rDNA and the promoter region of *At1g76930*, *At2g41260*, and *At5g46900* genes. For 45S rDNA, *At2g41260*, and *At5g46900*, there are substantial increases in the *ros1* mutant in methylation at CpXpX sites (Figure 4A), and some unmethylated CpXpX sites in the wild-type become methylated in the *ros1* mutant (Figure S4). There is also a large increase in *ros1* mutant plants in CpXpG methylation at the *At5g46900* promoter region and in CpG methylation at the *At2g41260* promoter region (Figure 4A). Compared to the wild-type, the *ros1* mutant has a dramatic

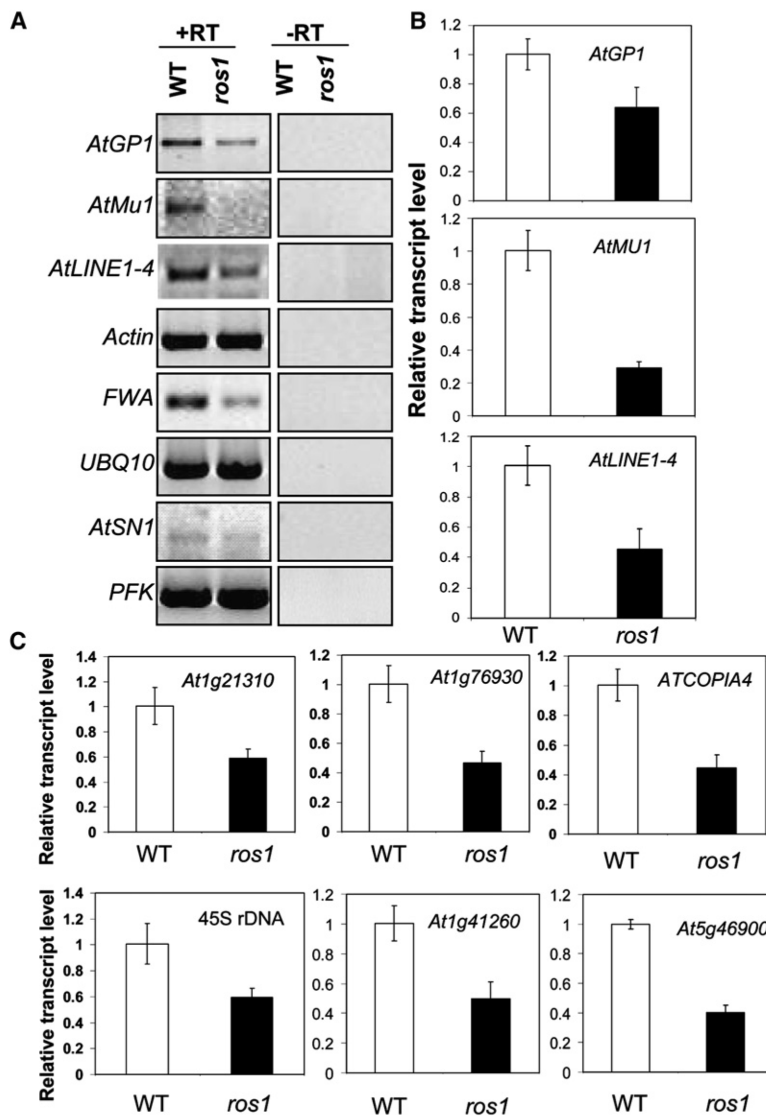

Figure 3. Analysis of Transcript Levels in the Wild-Type and the *ros1* Mutant

(A) Expression analysis by semiquantitative RT-PCR. Reverse-transcribed cDNA was amplified by PCR with primers for the indicated loci. Primers specific for *UBQ10* (ubiquitin extension protein 10) or *PFK* (phosphofructokinase β subunit) transcripts were used as internal controls. A parallel set of reactions without addition of reverse transcriptase (RT) were run as the control for genomic DNA contamination.

(B and C) Real-time PCR analysis of expression levels of indicated loci. Error bars indicate SD (n = 3–4).

increase in methylation at CpG, CpXpG, and CpXpX sites in the promoter region of *At1g76930* (Figure 4A). Most of the cytosines in this region are not methylated in the wild-type, but many of them become methylated in *ros1* (Figure 4B).

There is a growing interest in the plasticity of the plant epigenome as it responds to developmental and environmental cues [19–22]. It is likely that dynamic changes in DNA methylation status and histone-modification patterns are key to the plasticity and inheritance of the epigenome. Dynamic changes in DNA methylation require the actions of both DNA methyltransferases and demethylases [12, 18]. Through detailed bisulfite sequencing analysis of representative transposons, the *FWA* gene, and several other genes, we found an increase in primarily CpXpG and CpXpX methylation and found that many of the unmethylated non-CpG sites in the wild-type become heavily methylated in the *ros1* loss-of-function mutant. Associated with this increased methylation, these loci show decreased expression in the *ros1* mutant. At the present time, it is not known how ROS1 is targeted to specific loci and why the *ros1* mutation affects some but not other loci. Among the loci affected by *ros1*, a few (*RD29A* and *At1g76930*) are affected in cytosine methylation in all sequence contexts, although many others are affected primarily in non-CpG contexts. The latter ones include transposons and other repetitive sequences like *FWA* and 45S rDNA. It seems that the CpG methylation patterns of these repetitive sequences are largely protected, and this may be important in preventing potentially harmful transposons from being overly active. It is possible that the CpG methylation of the repetitive sequences may only enter into pathways mediated by ROS1 or ROS1-like demethylases and be substantially demethylated under exceptional situations (such as under stress) so that these repetitive sequences can be activated. It is also possible that ROS1 may act differently in different cells to contribute to a variety of methylation patterns.

Our results suggest that active DNA demethylation is important in pruning the methylation patterns of the genome, and even the normally "silent" transposons and other sequences are under dynamic control in the wild-type by methylation and demethylation. This dynamic

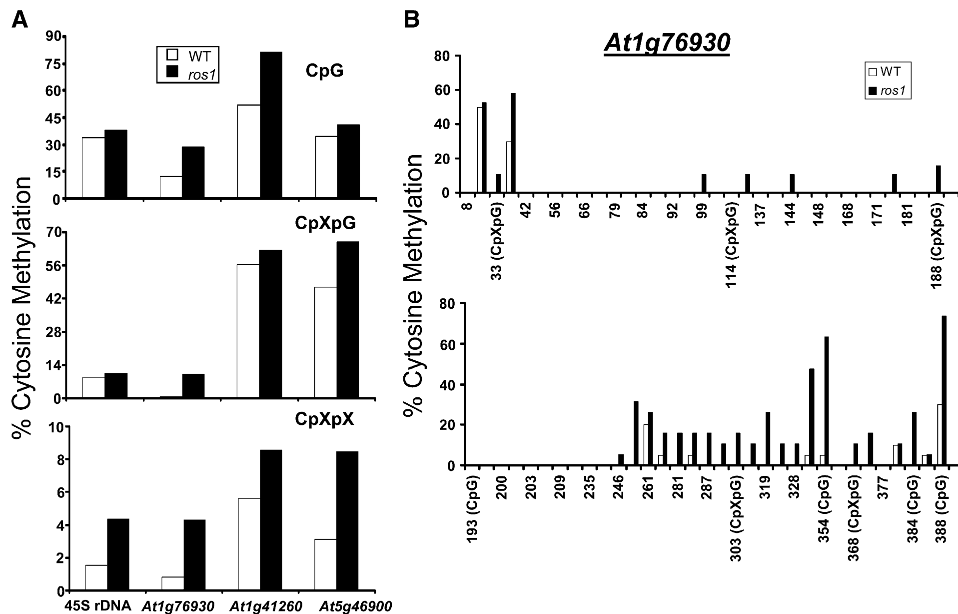

Figure 4. Comparison of DNA Methylation Levels between the Wild-Type and the *ros1* Mutant

(A) Analysis of CpG (top), CpXpG (center), and CpXpX (bottom) methylation by bisulfite sequencing. The sequences analyzed are indicated at the bottom. X could be A, T, or C.

(B) Detailed cytosine methylation profiles in the promoter region of *At1g76930* were revealed by bisulfite sequencing. Cytosine positions and sequence contexts (CpG and CpXpG; CpXpX not indicated) are indicated on the x axis.

control may be important in keeping the plant epigenome plastic or adaptable so that it can efficiently respond to developmental or environmental cues.

Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/1/54/DC1/.

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